PATENT

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Applicant(s): Brian J. Balin et al.

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For: TREATMENT AND DIAGNOSIS OF ALZHEIMER'S DISEASE

SECOND DECLARATION UNDER 37 CFR 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Brian J. Balin, hereby declare that:

1. I am a named inventor of U.S. Patent Application 09/227,749 filed January 8, 1999 and U.S. Provisional Application 60/070,855, filed on January 9, 1998.

2. A conception of the invention in my mind is shown by the attached Exhibit A, a manuscript entitled "Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain."

3. Exhibit A is a manuscript which provides data supporting the concept that *Chlamydia pneumoniae* is identified in the brains of typical patients with Alzheimer's disease (AD) and, as such, plays a role in its pathogenesis. Specifically, 19 late-onset AD brains and 19 non-AD brains were screened for the presence of *C. pneumoniae* DNA by two PCR assays. The results showed that 17/19 AD brains were positive for *C. pneumoniae* DNA in the PCR assays and 18/19 non-AD brains were negative for *C. pneumoniae* DNA in the PCR assays.(See, e.g., pages 7-8 of the manuscript.) Also, in order to identify the area(s) and host cell types within which the *C. pneumoniae* resides in the AD brain, immunohistochemical analysis was performed with a *C. pneumoniae* MOMP-specific monoclonal antibody on tissues from affected regions of AD brains. In sections of hippocampus and temporal cortex from 10 AD brains, a consistent

pattern of immunolabeling was observed. In sections of hippocampus and temporal cortex from 6 non-AD brains, no significant immunolabeling was observed. (See, e.g., page 10 of the manuscript.)

4. The dates that the manuscript referred to above was last modified and created are shown in the "Properties" option of the WordPerfect electronic copy of the manuscript. The properties option was printed and those 2 dates were redacted, resulting in Exhibit B attached to this Declaration. Both dates are earlier than May 6, 1997.

5. Labels "Exhibit A" and "Exhibit B" are shown in Exhibits A and B, respectively. The labels were applied to the Exhibits at locations that were blank on the originals.

6. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: April 11, 2005

Brian J. Balin

Attachments: Exhibits A and B

Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain

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Running Title: *C. pneumoniae* in Alzheimer's Disease

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(Abstract)

This study provides evidence of an association between infection with *Chlamydia pneumoniae* and sporadic late-onset Alzheimer's Disease (AD). Recent reports on the potential role of *C. pneumoniae* in atherosclerosis, and new studies correlating atherosclerosis with AD lead us to believe that *C. pneumoniae* also may be linked to AD. We screened nucleic acids prepared from affected/unaffected regions of AD and non-AD control brains for DNA sequences from the bacterium *Chlamydia pneumoniae*. These PCR analyses showed that brain areas with typical AD-related neuropathology, including hippocampus and temporal cortex, were PCR-positive in 17/19 AD patients. Similar analyses of identical brain areas of 18/19 control patients, including 3 with multiple sclerosis, were PCR-negative. Electron and immunoelectron microscopic studies of tissues from affected AD brain regions identified chlamydial elementary and reticulate bodies, but similar analyses of non-AD brains were negative for the bacterium. Immunohistochemical analyses of AD brains, but not those from controls, identified *C. pneumoniae* within pericytes near blood vessels, and in microglia and astroglia not associated with vessels. Further immunolabelling studies confirmed the organisms' intracellular presence primarily in areas of neuropathology in the AD brain. RT-PCR assays using total RNA from affected areas of AD brains demonstrated that transcripts from two *C. pneumoniae* genes were present. Thus, *C. pneumoniae* is present and apparently transcriptionally active in areas of neuropathology in the AD brain, suggesting that this organism might play some direct or indirect role in the pathogenesis of AD.

(Introduction)

Alzheimer's Disease (AD) is a severe and relatively common mental health problem in the US, affecting some 3-4 million people[1,2]. Research over the last decade has established that AD appears in two distinct manners: an early-onset form and a late-onset, sporadic form. Incidence of the latter increases with age, and AD is thought to be the most important single cause of senile dementia. Estimates of the prevalence of late-onset AD based on epidemiological evidence vary, in part due to the difficulty in diagnosis of the disease[1], but studies have indicated that at least half the total number of cases of dementia in the elderly can be attributed to AD[3,4]. The incidence of AD appears to vary with the population examined[5,6], but estimates as high as nearly 50% in those aged 85 yr and older have been reported[7]. Thus, AD is a significant mental health concern, and it will increase in importance with the continued aging of the population.

The detailed causes underlying the signature neuropathology seen in essentially all AD patients, *i.e.*, the neurofibrillary tangles (NFT), neuropil threads (NT), and neuritic senile plaques (NSP) comprised of deposits of *tau* protein and β-amyloid peptide (Aβ), respectively, are poorly understood. *tau* is a normal component of the neuronal cytoskeleton, and current evidence suggests that its abnormal deposition in NFT, NT, and dystrophic neurites results from post-translational modification of the protein. These modifications may take the form of abnormal phosphorylation, glycosylation, and/or transglutaminase-catalyzed covalent crosslinking[8-12]; deposition may also result from production of aberrant forms of the polypeptide

itself *via* alternate, unusual splicing of the messenger encoding it[13]. It is not clear whether NFT are primary lesions in AD, or whether their formation is a response to other neuronal injuries (reviewed in ref. 14). However, several reports have suggested that the level of NFT accumulation correlates most closely with the degree of cognitive impairment in AD patients[15].

Aβ peptide deposition appears to be a necessary step in the neuronal degeneration observed in AD[16-18]. In the familial form of AD (FAD), mutations in *βAPP* are associated with increased Aβ deposition and the early, severe onset of symptoms; such mutations may favor production of the Aβ42 form of the peptide with its increased tendency to aggregate[19]. Importantly, recent studies have shown that mutations in the genes encoding presenilin-1 (*PS-1*) and presenilin-2 (*PS-2*) lead to increased deposition of Aβ42 in FAD patients, and in an animal model system[20-22]. Mutations in *PS-1* and *PS-2*, with those in *βAPP*, appear to account for most early-onset, FAD cases[23-24]. Sporadic, late-onset AD is significantly more prevalent than early-onset FAD, and while the latter is almost certainly genetically-based, the former is not. One risk factor identified for late-onset disease is the homozygous or heterozygous presence of the *APOE* ε4 allele[18,25]. Not all patients with one or two ε4 alleles develop AD, but its presence does increase risk for the disease several-fold[26]. While possession of the ε4 allele has been associated with earlier onset and more rapid progression in FAD, one study suggested that the allele is a risk factor primarily in individuals younger than age 70 yr, and that it may be involved in as few as 10% of late-onset AD cases[27]; another study suggested that ε4 alone is not a risk factor for AD, but rather that it, in combination with infection by herpes simplex virus type 1 (HSV-1), constitutes a risk factor[28]. The ε4 protein does not appear to affect *βAPP*[29] or *PS-1* expression[30], but it has been reported to bind with higher affinity *in vitro* to Aβ than do the products of the two most common *APOE* alleles, ε2 and ε3[31]; ε4 binds to *tau* in some *in vitro* studies and may be present in NSP[32,33].

The neuropathology characteristic of both early- and late-onset AD is similar, and a final common pathway to that pathology cannot be dismissed; however, the detailed processes and causes initiating the pathology remain to be elucidated for late-onset disease[34,35]. Neurologic disease can be caused by bacteria and viruses, and infection with many agents that do not target the nervous system directly can elicit neuropathologic side-effects[36,37]. In large part because of this, several groups have attempted to establish a causal relationship between viral infection and development of AD, but to date no such etiologic link has been unequivocally demonstrated. For example, measles virus, various lentiviruses (including HIV-1), adenovirus, influenza virus types A and B, and many others have been investigated as potential agents associated with late-onset AD, and all have been dismissed[38-40]. Similarly, some bacterial species have been studied, including *Chlamydia trachomatis* and *Coxiella burnettii*, but none has provided an indication of a relationship with AD[41,42]. As mentioned, a recent study identified HSV-1 infection as a risk factor for development of AD in people possessing the *APOE*-ε4 allele[28], although it is not at all clear how and under what circumstances HSV-1 might interact with the ε4 allele or its gene product to produce or promote disease. In addition to viruses and bacteria, unconventional agents (*i.e.*, prions) have been considered in the pathogenesis of AD and discarded[43,44]; the possible roles of environmental factors, including diet and extended/acute exposure to aluminum, also have been investigated and have provided no clear evidence of association (reviewed in refs. 2, 45).

Chlamydia pneumoniae is an obligate intracellular bacterium that, in contrast to its well-known sister species *C. trachomatis*, is a respiratory pathogen, initially infecting the oral and nasal mucosa[46,47]. This organism has been shown to be a significant agent in acute respiratory infections, including pneumonia, sinusitis, and bronchitis[47,48]. Recent studies have implicated *C. pneumoniae* in more severe and chronic pulmonary pathologies, including sarcoidosis, adult

onset asthma, and chronic obstructive pulmonary disease[48-51]. Epidemiologic analyses have shown that prevalence of infection with *C. pneumoniae* is high in all populations studied, and that it increases with increasing age[48,52]. In the Western world where population densities are relatively low, children under the age of 5-10 yr rarely show anti-*C. pneumoniae* antibodies (Ab), but incidence rises sharply thereafter; in higher density populations such as those found in Japan, childhood infection with *C. pneumoniae* seems to be more common than in the West[48,52]. As mentioned, Ab titers against *C. pneumoniae* increase with increasing age, peaking in the 6th-7th decades and beyond; *e.g.*, in one study, males 60 yr and older in Seattle showed a prevalence rate of 70%[48,52]. All studies indicate that virtually everyone is infected with *C. pneumoniae* at some during his/her lifetime, and that reinfection is common[52].

Interestingly, infection with *C. pneumoniae* has been implicated in several surprising clinical manifestations, including meningoencephalitis, heart disease, acute myocardial infarction, and atherosclerosis[53-60]. While a causal relationship between infection with *C. pneumoniae* and the latter has not been unequivocally established, many studies have noted a significant correlation between serum anti-*C. pneumoniae* Ab titers and coronary artery disease; the bacterium has been identified by electron microscopy and other methods in atheromatous plaques (see refs. 60-61 for review). Moreover, the organism has been associated with infections of the central nervous system in serological studies[59], and with cerebrovascular disease[62]. One recent report has postulated a relationship among atherosclerosis, *APOE* gene products, and late-onset AD[63]. For all these reasons, as well as its ubiquity in older adults, we investigated a possible relationship between infection with *C. pneumoniae* and sporadic AD. Using polymerase chain reaction (PCR) assays, we demonstrate here that chromosomal DNA from the organism is present in brain areas showing neuropathology in the vast majority of AD patients examined, but not in similar brain regions

of age-matched non-AD control patients. Using immunocytochemistry, electron-, and immunoelectron microscopy (EM, IEM), we further show that the organism is found primarily at sites in which typical AD neuropathology is evident. The studies presented identify the host cells harboring the organism in the AD brain as pericytes, microglia, and astroglia, and analysis of transcriptional activity of *C. pneumoniae* in AD brain tissue suggests that the organism is viable in this context.

(Results)

C. pneumoniae chromosomal DNA in the Alzheimer's brain

To examine whether *C. pneumoniae* is present in the brains of late-onset AD patients, we employed two independent PCR assays to assess chromosomal DNA from the organism in post-mortem brain tissues from 19 late-onset AD and 19 non-AD control patients. From each AD and control patient, we screened tissue from one or more brain area expected to show AD-related neuropathology, as well as from the cerebellum, a region normally less-affected/unaffected. As an internal control, nucleic acids from all samples were also screened by PCR for DNA from other bacteria, including *C. trachomatis*, *Borrelia bergdorferi*, *Mycoplasma pneumonia* and *M. hominis;* all samples were negative for each of these (data not shown). A summary of relevant information for all patients is given in Tables 1-2. Determination of *APOE* genotype for each patient showed that 11/19 AD patients (58%) had at least one copy of the ε4 allele, and two patients were homozygotes; this allele was present in only 4/19 controls (21%; see Discusssion). All samples but one (Pcx, patient C1) from non-AD patients were negative for *C. pneumoniae* DNA sequences in PCR assays, including those from patients with multiple sclerosis (Table 1). Of the 19 late-onset AD patients, samples from 17

(90%) were positive in both PCR assays when nucleic acids from hippocampus, temporal cortex, and/or other areas were assayed (Table 2). Nucleic acids from cerebellum of 4 AD patients (AD9, AD10, AD16, AD19) were also PCR-positive for *C. pneumoniae* DNA; each of these was determined to have severe neuropathology by histological examination of tissue. All samples from affected brain areas of 2 AD patients (AD3, AD5) were PCR-negative for *C. pneumoniae* in PCR assays; this may reflect sampling error, but microscopic examination indicated that these 2 brains showed far less severe neuropathology than did all other AD brains studied (see Discussion). Thus, PCR screening indicates that DNA sequences from *C. pneumoniae* are common in areas of the AD brain exhibiting disease-related neuropathology, but are more rare in unaffected areas, in a large majority of patients studied; such sequences are extremely uncommon in congruent brain regions from non-AD patients of similar age, including those with an unrelated neurological disease.

Ultrastructural localization of C. pneumoniae in the Alzheimer's brain

To confirm these striking PCR screening results and to identify the organism in affected CNS regions, we analyzed brain sections from AD and non-AD control patients using EM and IEM. In all species of *Chlamydiae*, the extracellular, infectious form of the organism (elementary body, EB) alternates with the intracellular, vegetative growth form of the organism (reticulate body, RB)[64]. Survey of tissue sections at the ultrastructural level revealed that areas of the hippocampus, temporal cortex, and/or other regions of AD brains showing neuropathology contained structures whose morphology was consistent with that of both EB and RB (Fig. 1)[65]. These chlamydia-like structures were localized in variable-sized inclusions within the neuropil (*e.g.*, Figs. 1A, 1B, 1E). Often, a distinct membrane with electron dense nuclear material could be distinguished, and the pear-shaped EB morphology typical of some *C. pneumoniae* strains

could be observed (Fig. 1B)[65]. We frequently identified structures resembling RB in the process of cell division (data not shown; see below). The sizes of the chlamydia-like bodies observed ranged from 0.2 to 1.3 μm in diameter, falling within the size range characteristic for typical *C. pneumoniae* EB and RB[82]. The data provided in Fig. 1 are representative of the 10 AD brains subjected to EM analysis; we found no similar objects in any tissues subjected to the same analysis from 2 control patients, except sections from parietal cortex of patient C1 (data not shown). Thus, ultrastructural studies of areas of AD brains showing neuropathology, but not congruent areas from control brains, demonstrate forms consistent in size and morphology with those typical of *C. pneumoniae*.

To confirm that the chlamydia-like bodies observed were in fact *C. pneumoniae* cells, we

employed IEM to examine several brains that were PCR-positive for the organism, and which had been analyzed by EM. For these analyses, we used a monoclonal Ab targeting the major outer membrane protein (MOMP) of *C. pneumoniae*; organisms binding that Ab were visualized *via* 5 nm and 15nm gold-conjugated secondary Ab to the anti-MOMP primary Ab. In all these analyses, background labelling was extremely low, and examination of sections from PCR-negative non-AD patients showed no significant or specific labelling at all (data not shown). Representative results of IEM studies are included in Fig. 1. The anti-MOMP primary Ab labels EB (Fig. 1C) and RB (Fig. 1D), thus confirming both developmental forms in the AD brain. Organisms showed variable distribution of immunoreactivity including partial/complete circumferential labelling, and immuno-labelled bacteria were observed often in sections displaying neurodegenerative changes typical of AD (see below). The only normal component seen in tissue sections which resemble the objects shown in Fig. 1 were lysosomal dense bodies within dystrophic neurites, but these were never labelled with the anti-MOMP Ab. Thus,

IEM analysis of tissues from affected brain regions of AD patients confirms that *C. pneumoniae* is present in those tissues; such bacterial cells are absent in materials from congruent brain regions of non-AD patients.

Immunohistochemical localization of C. pneumoniae in the Alzheimer's brain

To identify area(s) and host cell types within which the bacterium resides in the AD brain, we performed immunohistochemical analysis of tissues from affected regions of AD brains, and congruent regions from non-AD control brains. In these analyses we used two Ab; the first was a genus-specific monoclonal Ab targeting the LPS of the organism, and the second was the *C. pneumoniae* MOMP-specific monoclonal Ab employed in IEM studies. In sections of hippocampus and temporal cortex from AD brains, a consistent pattern of immunolabelling was observed in perivascular regions of small/medium sized blood vessels in the neuropil (Figs. 2A, 2D). In most sections, specific labelling also appeared in microglia- and astroglia-like cells in the vicinity of, or distant from, blood vessels (Figs. 2B, 2E). In sections of hippocampus and temporal cortex from brains of the 6 non-AD patients examined, including MS patients, no significant immunolabelling was observed using either the anti-LPS and anti-MOMP Ab (Figs. 2C, 2E). We also examined congruent brain sections from the 2 AD patients PCR-negative for *C. pneumoniae* (AD3, AD5; Table 2), and both were immunonegative for the organism even though they showed mild AD-related neuropathology. Staining of AD brain sections sometimes revealed cellular processes, presumably from pericytes (see below), enclosing or abutting the abluminal surface of blood vessels. The pattern of immunoreactivity was consistent from brain to brain in sections from the 10 AD patients examined. We found some variability among brains in overall level of immunopositivity; this probably reflected a difference in bacterial load in the tissues examined but may also reflect sampling irregularity. Thus, immunohistochemical

analysis of tissues from affected AD brain regions confirmed the presence of *C. pneumoniae* within cells encircling blood vessels and cells adjacent to, as well as distant from, those vessels.

Host cells for *C. pneumoniae* in the Alzheimer's brain, relationship to neuropathology

If *C. pneumoniae* is involved in the neuropathogenesis of late-onset AD, then an understanding of specific host cell types harboring the organism in the brain, and their relationship to AD pathology, is required. We used double immunolabelling to identify the chlamydia-bearing cell types observed, and to investigate the relationship between infected cells and NFT/NSP. GFAP is a standard marker for astroglial cells[66]. Double labelling of tissue sections from several AD brains using polyclonal anti-chlamydial LPS Ab and a monoclonal anti-GFAP Ab demonstrated that this cell type is a common host for *C. pneumoniae* in the AD brain. Fig. 3 presents a typical double immunolabelling result; cells identified by the polyclonal anti-chlamydial LPS Ab, illuminated in Fig. 3A using an FITC-conjugated secondary Ab, co-localize with cells expressing GFAP, identified in Fig. 3B by DAB development. Similarly, iNOS is an enzyme produced extensively by activated microglia[67-69]. Fig. 3 presents a section from an AD brain in which the anti-MOMP Ab has been used to identify *C. pneumoniae*-containing host cells (Fig. 3D); the same cells are stained strongly with the anti-iNOS Ab, immunoreacted with an FITC-conjugated secondary Ab (Fig. 3C). Thus, host cells identified in studies presented in the previous section as residing either near blood vessels or distant from them are indeed astroglia and activated microglia, as suggested by their morphological characteristics. The chlamydia-infected host cells visualized as surrounding blood vessels in the AD brain are probably pericytes, as strongly suggested by data in Figs. 2A and 2D. No single surface protein characterizes these cells; however, we used the anti-iNOS Ab in combination with the anti-MOMP Ab to identify this cell

type, since expression of this enzyme is induced in infected pericytes (data not shown)[70]. In addition, pericytes constitutively express ICAM-1[71], an adhesion protein, and double labelling with the anti-chlamydial LPS Ab and a monoclonal Ab targeting ICAM-1 also indicated that the chlamydia-infected cells surrounding blood vessels include pericytes (not shown). Thus, at least three cell types, astroglia, microglia, and pericytes, harbor *C. pneumoniae* in the AD brain.

We considered it important to determine whether chlamydia-infected cells are concentrated primarily at sites in which localized AD-related neuropathology is evident, or whether the organism is found generally throughout AD brain tissue. The PHF-1 monoclonal Ab was used as a marker to identify neuritic pathology in tissue sections from the AD brain[72]. Fig. 4 presents typical consecutive tissue sections from the temporal cortex of an AD patient labelled with the anti-PHF-1 and anti-MOMP Ab. Staining with the latter (Fig. 4A) demonstrated the common presence of chlamydia-infected cells in the vicinity of PHF-*tau* protein deposition in neurites within NSP (Fig. 4B); immunolabeled cells in Fig. 4A are primarily glial. Similar labelling studies of AD brain areas showing few/no NSP showed that virtually no host cells harboring *C. pneumoniae* were present; examination of sections from non-AD brains identified no infected cells (data not shown). Thus, chlamydia-infected cells in the AD brain are concentrated in regions of neuropathology.

Transcriptional activity of C. pneumoniae in the AD brain

Previous work from this laboratory investigating the relationship between infection with *C. trachomatis* (Gérard *et al.*, submitted) or *C. pneumoniae* (Gérard *et al.*, submitted) and development of reactive arthritis indicated that both species are transcriptionally active in their synovial context. Those studies employed freshly-procured synovial biopsy samples. Recent research has indicated that, in some cases, RNA can remain intact in brain tissues for several

hours post-mortem[28,73], and to obtain some insight into whether *C. pneumoniae* is transcriptionally active during CNS infection of AD patients, we performed RT-PCR assays targeting mRNAs from the *C. pneumoniae* gene encoding the KDO transferase enzyme required for bacterial LPS synthesis, and that specifying the Mr=72000 heat shock protein (*hsp72*). We prepared RNA from hippocampus and/or temporal cortex, and cerebellum of 2 patients (AD2, AD14) and analyzed it for these *C. pneumoniae* messengers. The results given in Fig. 5 demonstrate that RNA from brain regions showing neuropathology contained these bacterial transcripts; in contrast, RNA from cerebellum, a region PCR-negative for *C. pneumoniae* DNA in the two patients studied here (Table 2), was negative for both messengers, as expected. Thus, *C. pneumoniae* expresses at least some genes during CNS infection of AD patients, suggesting that such infections involve vegetatively-growing bacteria.

Discussion

Chlamydia pneumoniae is an obligate intracellular respiratory pathogen that has been associated with several unusual pathologies, including some related to function of the nervous system[59,62]. Importantly, infection with the organism has also been linked to development of atherosclerosis, a disease recently reported to be associated with development of late-onset AD[63]. These observations, in combination with others indicating increased prevalence of *C. pneumoniae* infection with age, suggested to us that this organism might be associated with development of sporadic AD. In the work presented here, we demonstrate that chromosomal DNA from the bacterium is present in brain tissues from areas showing typical neuropathology in a large majority of AD patients, and that it is absent in virtually all samples from congruent brain regions in non-AD control patients. RT-PCR assays targeting mRNAs from two *C.*

pneumoniae genes suggest that the organism is transcriptionally active during CNS infection. Ultrastructural analyses of AD brain tissues PCR-positive for the bacterium identified chlamydia-like bodies in both EB and RB form, and these were confirmed to be *C. pneumoniae* by IEM using a chlamydial MOMP-specific Ab. Immunohistochemical analyses of AD brain tissues using the anti-MOMP and an anti-chlamydial LPS Ab showed that cells both adjacent to and distant from the vasculature are commonly infected, and double immunolabelling studies demonstrated that these host cell types include microglia, astroglia, and pericytes. Significantly, immunolabelling of serial tissue sections from affected AD brain regions with the PHF-1 and anti-chlamydial LPS Ab showed that chlamydia-infected host cells are concentrated primarily in areas of neuritic pathology. Together, these results indicate that metabolically-active *C. pneumoniae* are found in the AD brain, specifically in regions showing neuropathology.

Pneumonia is a common cause of death among AD patients[74,75], although one study indicated that it may be more prominant among those with the most advanced dementia[76]. In the AD patient group available to us for study, proximal causes of death included many of those normally seen in advanced age, with pneumonia a relatively rare occurence; indeed, causes of death do not appear to be significantly different between our control and AD patient groups (Tables 1-2). We do not have an assessment of level of dementia in the three AD patients for whom pneumonia was the proximal cause of death, or for any other patients studied here, nor do we have access to detailed clinical records to know whether any of these patients had pneumonia prior to death. Regardless, our PCR screening system showed *C. pneumoniae* DNA to be present in brain in an extraordinarily high proportion of AD patients (90%). All control and AD brain samples available were screened by PCR, of course, and most AD, along with selected non-AD control, specimens were also examined by EM/IEM and/or immunohistochemical methods, when enough tissue was available to do so; results of these

latter analyses were always consistent with PCR data and internally consistent among samples. We could not examine tissues other than brain from any of our AD or control patient, since such specimens were not available, and thus we do not know whether dissemination of *C. pneumoniae* is general in individuals with/without AD who are infected with the organism. However, it seems unlikely that dissemination to the brain is a common occurrence with age, since we found evidence of *C. pneumoniae* in one brain region, but not others, from only one non-AD patient. It would be of some interest to assess a larger population of non-AD patients for presence of the organism in brain, and other organ systems, as a function of increasing age. The critical question of whether CNS infection with *C. pneumoniae* is directly responsible, either partly or fully, for the neuropathogenesis of AD, or whether the organism is simply an opportunistic invader of an organ already damaged by other means, therefore remains open.

The data presented here do not establish a causal relationship between acute or chronic infection with *C. pneumoniae* and development of sporadic, late-onset AD. Rather, our observations simply demonstrate that many cells in areas of the AD brain showing typical neuropathology harbor the organism in a high proportion of patients. Aside from the issue of whether chlamydial infection of the brain is directly responsible for AD-related pathology, however, such infections may explain some characteristics of the disease. For example, inflammation is common in the AD brain in areas showing neuropathology, and this inflammation, currently thought to result from Aβ deposition, has been advanced as a pathogenic mechanism in the disease[77,78]. Indeed, inflammation has been implicated as an important factor in a number of diseases, including stroke and hypertension[79], and some studies have indicated that administration of non-steroidal anti-inflammatory drugs is beneficial in the treatment of AD[80-83]. Chlamydial infection engenders a strong inflammatory response[48,52,84], and infection by *C. pneumoniae,* therefore, may be partly responsible for the

inflammation observed in the AD brain. In this respect, it is of interest that our results demonstrate the frequent infection of microglia and astroglia with *C. pneumoniae* in the AD brain. Microglia are the resident tissue macrophages of the brain, and once activated they can be a source of inflammatory cytokines, including IL-1β, TNFα, IL-6, and others[85]; astroglia also produce proinflammatory cytokines[86]. We made no attempt to assess relative numbers of microglia, astroglia, and pericytes infected with chlamydia in the work presented here, but the predominant CNS host cell type appears to us to be glial. Our results clearly indicate that chlamydia-infected glial cells are concentrated in areas of neuropathology in the AD brain, areas which usually show inflammation, strongly suggesting a relationship between that pathology and producers of relevant cytokines. In addition, it would be of interest to know whether chlamydia-infected microglia, astroglia, and/or pericytes are involved in aberrant production/deposition of Aβ, perhaps *via* alteration of *βAPP*, *PS-1*, or *PS-2* expression, or modification of processing of the *βAPP* gene product. We are now exploring these possibilities using appropriate cell lines infected with *C. pneumoniae*. If the bacterium is, in fact, responsible for/contributory to the pathogenesis leading to AD, access of *C. pneumoniae* to the brain *via* the circulatory system also may aid in explaining the as yet poorly understood regional deposition of NFT and NSP observed in the AD brain.

Previous studies of other potential microbial pathogens in the AD brain have indicated no relationship between infection and disease, with the exception of HSV-1; infection with this virus in the presence of the *APOE* ε4 allele was suggested to be a risk factor for AD in the large number of patients examined in the study, but the percentage of AD patients showing HSV-1 DNA sequences in brain was lower than that reported here for *C. pneumoniae*[28]. Our assessment of *APOE* genotype for the control and AD patient groups is consistent with it being a risk factor for the disease, especially in combination with chlamydial infection of the brain.

The presence of at least one ε4 allele in most AD patients shown to be PCR-positive for *C. pneumoniae* in our study may suggest that this gene product allows, or even promotes, CNS infection; this contention seems to be consistent with results from the study showing the somewhat lower level of correlation between HSV-1 DNA and the ε4 allele in the AD brain[28]. More study will be required to assess this possibility, but it is relevant to note that the two AD patients studied here who were PCR-negative for *C. pneumoniae* were ε3 homozygotes. As mentioned, these had the least severe neuropathology of our AD patient group. The four patients with the most severe neuropathology showed chlamydial DNA in all the expected areas and in cerebellum; three of these latter four patients possessed an ε4 allele.

Immunohistochemical staining clearly showed a large number of infected cells associated with blood vessels in the brain, possibly suggesting that dissemination of the organism from the site of primary infection involves the vasculature. In studies unrelated to those here, we showed that the principal host for *C. trachomatis* are monocytes/macrophages in persistent synovial infection[87]; this indicated that dissemination from the genital system to the joint might be *via* blood, and we have recently demonstrated the presence of this chlamydial species in blood monocytes from patients with early arthritis[88]. No data bearing on the means of dissemination of *C. pneumoniae* to the brain are currently available, of course, but the organism was identified in circulating mononuclear cells in a rabbit model of infection with the organism[89]. Blood-borne transport of *C. pneumoniae* may not require an infected host cell, however. Rather, the infectious EB form of the organism can be a means by which circulatory dissemination to the brain occurs. The means by which EB, or an infected cell, might pass the blood-brain barrier remains to be determined, but sufficient evidence suggests that this barrier is affected in AD patients[90].

We have considered, and to some extent investigated, another route by which the

bacterium might reach the CNS from the olfactory/nasal mucosa. While the distribution and overall density of NSP, NFT, and NT vary among the usual affected areas of the AD brain, and among individuals, one region which is universally affected in all AD patients is the hippocampus. Fibers from the olfactory bulb terminate in the piriform cortex, from which secondary connections terminate in the amygdala and adjacent entorhinal cortex. Entorhinal neurons form the perforant pathway, which communicates with the adjacent hippocampal formation; all these areas routinely exhibit damage in the AD brain. The bacterium might reach the hippocampus indirectly following passage from the nasal mucosa, olfactory nerve, olfactory bulb and tract. The olfactory bulb is not normally obtained or analyzed during standard autopsy procedures at our institution, but we were able to procure this tissue from two AD patients studied here, and screen for *C. pneumoniae* DNA using the PCR assays; both olfactory bulbs were PCR-positive. We do not know from this initial observation whether the direction of spread of infection is from olfactory bulb to hippocampus or *vice-versa*, but this may be an important area for further investigation.

An unequivocal pre-mortem diagnosis of AD is notoriously difficult to establish, and during the course of this work we were often asked whether serologic analysis of potential AD patients might be useful in establishment of such diagnoses. We doubted that it would, given the known increase in incidence of anti-*C. pneumoniae* Ab with increasing age[48,52]. Nonetheless, we undertook a small study to determine presence/titers of serum anti-*C. pneumoniae* IgG in a group of 6 non-demented, healthy control patients 70 yr or older, and an equal number of presumed AD patients of the same ages. As expected 5/6 controls were positive for the Ab, indicating that simple presence of anti-*C. pneumoniae* IgG in a given patient will not be useful in diagnosis. Interestingly however, of the 5 IgG-positive control subjects, Ab titers in all but one were low, with a single individual possessing a titer suggestive of

active/recent infection with the organism. Anti-*C. pneumoniae* IgG was present in all but one putative AD patient, again as expected, but Ab titers in this group were uniformly higher than those of. Thus, while assessment of far more patients will be required to determine if anti-*C. pneumoniae* Ab titers can give insight concerning AD status, such an approach may prove to be a useful diagnostic adjunct.

Although we have not established a causal relationship between CNS infection with *C. pneumoniae* and development of late-onset AD, localization of C. pneumoniae in glial cells within regions of AD neuropathology mandates further study to determine whether or not a causal relationship exists. Regardless, the results presented here do indicate that CNS infection by *C. pneumoniae* should be considered a risk factor for late onset AD, especially in individuals possessing at least on APOE e4 allele. It will be important to determine whether, in the case of late-onset disease, some genetic predisposition exists in AD patients for spread of *C. pneumoniae* to the CNS. Furthermore, it will be of significant interest to examine specimens from patients with other neurodegenerative diseases to determine whether dissemination of the bacterium is common among such patients, or whether it is confined to those with sporadic, late onset AD.

Methods

Patient samples. Postmortem tissue samples from various brain regions of patients with or without AD were obtained from the Harvard Brain Tissue Resource Center, through Dr. Gail Johnson of the University of Alabama (Birmingham) Brain Resource Center, through Dr. William Hill of the Medical College of Georgia (Augusta GA), and from the MCP-Hahnemann School of Medicine Dept. Pathology. All samples from patients indicated as having AD were confirmed as AD by histopathologic examination by a certified

neuropathologist, using standard criteria (NINDS/CERAD[64]). All AD patients studied had late-onset disease. Samples from non-AD patients were age-matched as well as possible to those of AD patients, and each set of such samples was examined histologically for NSP and NFT and confirmed as non-AD. Average age of control patients was 72.6 yr; that of AD patients was 77.7 yr. All AD/non-AD brain samples were screened by PCR analysis. Virtually all AD, and most control, samples were also examined by EM, IEM, or immunohistochemistry, although each could not be subjected to every analysis due to limited availability of tissues. Table 1 summarizes clinical information for control patients from whom samples were obtained, and Table 2 provides congruent data for AD patients.

Preparation and analyses of nucleic acids. Nucleic acids were prepared from tissue samples as described[65-67], and 1 μg nucleic acid mixture was used in each PCR screening assay. EB of *C. pneumoniae* strain TW183 were obtained from the ATCC, and DNA was prepared from them for control amplifications[67]. PCR assays to screen for *C. pneumoniae* chromosomal DNA targeted two genes, each in an independent system. In one, primers targeting the 16S rRNA gene were used as described[68]. The second assay targeted the chlamydial MOMP gene and used primers designed using GeneRunner® software (Hastings Software, Hastings NY); Alan, are these nested primers, and if they are which are inner and which are outer ??? those primer sequences were derived from bases 26-43 and 445-463 of the *C. pneumoniae* MOMP coding sequence[69] and were analyzed for sequence specificity *via* "Blast" comparison with all DNA sequences in GenBank. Cycling for all PCR screening assays was done in a Barnstead/Thermolyne instrument, and products were analyzed on 2% agarose gels. Positive signal in the 16S rRNA-directed

assay is a band of 463 bp; that in the MOMP-directed assay is a band of 353 bp. All assays were done in duplicate by two different investigators on different days and in a fully blinded fashion. Results of screening for each sample were always consistent between the 16S- and MOMP-directed PCR assays. Primers for PCR assays targeting sequences from *C. trachomatis, Borrelia bergdorferi, Mycoplasma pneumoniae,* and *M. hominis* were as described[67,70]. *APOE* genotypes were determined as described[71].

For RT-PCR assays, RNA was prepared from total nucleic acids by digestion with RNase-free DNase1 (RQ1; Promega Biotech, Madison WI); purity of RNA was assessed by PCR amplification using primers given below in the absence of reverse transcription. RT reactions used 1 μg total RNA, were done in the standard manner[72], and employed nested primer systems for the *C. pneumoniae hsp72* coding sequence (outer: bases 160-184 and 1066-1086; inner: bases 373-392 and 799-822[73]) and the KDO transferase gene (outer: bases 132-152 and 946-965; inner: bases 527-549 and 797-815[74]). These primers were designed and their specificity confirmed as above. Positive signals for the *hsp72* and KDO transferase mRNAs were bands of 450 bp and 289 bp, respectively.

Electron microscopic and immunoelectron microscopic analyses. Brain tissues from AD/non-AD patients were immersion fixed in either 10% neutral buffered formalin or 4% paraformaldehyde in PBS and cut into 1 mm^3 blocks. Blocks were osmicated in 1% OsO_4 prior to propylene oxide/resin infiltration and embedment in Embed-812 (Electron Microscopy Sciences, Fort Washington PA). Sections were cut on a Sorvall Porter-Bloom MT2B ultramicrotome, post-stained with 2% uranyl acetate, and viewed/ photographed on a Zeiss 10 electron microscope.

For pre-embed IEM, 1 mm^3 blocks of tissue were fixed overnight in 4% paraformaldehyde with 0.05% glutaraldehyde, 0.1% saponin in 0.2 M phosphate buffer pH 7.0. After fixing, tissues were blocked for 2 hr in 100 mM ammonium chloride in 0.2 M phosphate buffer pH 7.0 followed by incubation with a monoclonal mAb (Washington Research Foundation, Seattle WA) specific for the OMP of *C. pneumoniae* diluted 1:10 in 0.2 M phosphate buffer pH 7.0, 5% cold water fish gelatin (CWFG; Sigma Chemical Co., St. Louis MO), at room temperature for 15 min. Following rinses in 0.2 M phosphate buffer containing 5% CWFG, 20 mM glycine, the tissues were incubated overnight at 4^0C with 15 nm gold-conjugated anti-mouse secondary Ab (Amersham Life Sciences, Arlington Heights IL) diluted 1:50 in 0.2 M phosphate buffer. Subsequently, they were rinsed in 0.2 M phosphate buffer and refixed in 2% glutaraldehyde solution, followed by immersion in 1% OsO_4 for 30 min at room temperature. The tissues were dehydrated in ethanols, contrast enhanced with 2% uranyl acetate, and processed for embedment in epon resin. Thin sections were prepared, viewed, and photographed as above. For post-embed IEM, thin sections of tissues embedded in Epon 812 were etched with 7% hydrogen peroxide for 60-75 sec and rinsed with milli-Q H_2O. Sections were further treated with citric acid buffer (pH 6) for 2 min, rinsed with milli-Q H_2O, and incubated for 1 hr in undiluted primary monoclonal anti-OMP mAb (RR402; DAKO, Carpenteria CA). Following a rinse in milli-Q H_2O, sections were blocked for 2 min with 1% CWFG in PBS and 0.1% acetylated-BSA (Auron). Sections were incubated with 15 nm gold-conjugated mouse secondary Ab (Amersham) diluted to 1:5 in 0.2 M phosphate buffer. Following this incubation, sections were rinsed, post-stained with 2% uranyl acetate, and viewed as above.

Immunohistochemical analyses. Formalin-fixed paraffin-embedded tissues were cut into 7-10μm sections and prepared for immunohistochemical analysis as described[11]. Briefly, following deparaffinization/rehydration, sections were blocked for endogenous peroxidase activity for 5-15 min. Antigen retrieval was enhanced by treatment with citric acid buffer at pH 6.0 followed by microwaving for 1 min. Following a rinse in ddH_2O, sections were blocked for 30 min to 1hr in 1-5% CWFG. Frozen sections (~10μm) were used to verify/complement the immunolabelling of formalin-fixed tissues. For these, sections were rinsed in 1.5T buffer (0.05M Tris pH 7.6, 1.5% NaCl) at room temperature and fixed for 20 min in ice-cold 4% paraformaldehyde in PBS (pH 7.4). After rinsing in 1.5T buffer, the tissue was blocked for endogenous peroxidase, before blocking for 1 hr with 5% dried milk solids in 1.5T buffer, and again with 1% CWTF. Sections from frozen and formalin-fixed tissues were incubated with a primary monoclonal mAb targeting the *C. pneumoniae* OMP (1:50-1:250 dilution) or from the Dako Corp. was used (1:5 dilution in 5% dried milk solids). In related studies, a monoclonal mAb targeting the bacterial lipopolysaccharide (LPS; 1:50-1:250 dilution in 5% dried milk solids; gift of Dr. S. Tirrell, Chiron Diagnostics), was used. In yet other studies, consecutive tissue sections were immunolabelled with monoclonal anti-OMP mAb and a monoclonal mAb targeting the PHF-*tau* protein[72] (PHF-1). In all cases, slides were incubated with primary mAb for 1-4 hr at 4°C, washed with 1.5T buffer at room temperature, incubated with goat anti-mouse or goat anti-rabbit secondary Ab (Amersham; 1:200 dilution in 5% dried milk solids) for a minimum of 1 hr at room temperature or overnight at 4°C. After rinsing, tissues were incubated with ClonoPAP (mouse or rabbit; Sternberger Inc., Baltimore MD; 1:200 dilution in 5% dried milk solids) at room temperature for 30 min. Slides were washed in 1.5T buffer before/after development with 0.05% DAB (Sigma Chemical Co., St. Louis MO) in 0.01% H_2O_2 for 8 min at room temperature. Tissues were dehydrated with ethanols/ xylenes and mounted in

permount.

For double immunolabelling, several Ab were used in conjunction with one another to identify cell types infected with *C. pneumoniae*. In one set of studies, a primary (anti-mouse) monoclonal mAb targeting glial fibrillary acidic protein (GFAP; SMI21, Sternberger) was used with a rabbit primary polyclonal pAb targeting the chlamydial LPS. In complementary studies, primary Ab employed were a rabbit polyclonal targeting inducible nitric oxide synthase (iNOS; Transduction Laboratories, Lexington KY) and the monoclonal anti-*C. pneumoniae* OMP mAb. Secondary Ab were either anti-mouse conjugated to HRP (1:200 dilution) specific for the monoclonals, or anti-rabbit IgG conjugated with FITC (1:20-1:50 dilution). In all double-immunolabelling experiments, primary Ab were added to sections at the same time, and rinses/reblocking were as above. Sections were incubated with secondary Ab overnight at 4°C, rinsed and treated with mouse Clonopap, and processed for immunoperoxidase as above. Sections were examined using an Olympus BX60 microscope with epifluorescence (courtesy Dr. D. Simon, Ms. K. Wallace).

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Table 1. Summary of control patient characteristics and chlamydia-directed PCR results

					PCR[1]					
Patient	Sex	Age at Death	Cause of Death[2]	Time PM (hr)	CB	HP	Tcx[3]	Other	*APOE*[4]	
C1 (9104)	f	74	respiratory failure	14			-	- -	Pcx+/-	ε3/ε3
C2 (9162)	f	58	multi-organ failure	4			-	- -		ε3/ε3
C3 (9210)	f	90	myocardial infarction	9	-	na	-		ε3/ε3	
C4 (9211)	m	57	cardiac arrest	11	-	-	-		ε3/ε4	
C5 (9260)	m	66	sepsis	8	-	-	-		ε3/ε4	

C6 (9147)	f	78	pulmonary disease	5	-	na	-		ε3/ε3
C7 (9227)	f	83	renal failure	3	-	na	-		ε3/ε3
C8 (9322)	m	71	aortic aneurism	6	-	na	-		ε3/ε3
C9 (9469)	f	81	respiratory failure	8	-	na	-		ε3/ε3
C10 (9471)	m	50	adenocarcinoma	10	-	na	-		ε3/ε3
C11 (9213)	m	68	adenocarcinoma	4	-	na	-		ε3/ε4
C12 (9231)	f	90	lacunar infarcts	4	-	na	-		ε3/ε4
C13 (9221)	f	84	pneumonia	7	-	na	-		ε3/ε3
C14 (295)	m	64	respiratory failure	10	-	na	na	Pfcx-	ε2/ε3
C15 (169)	m	74	respiratory failure	5	-	na	na	Pfcx-	ε3/ε3
C16 (250)	m	77	congestive heart failure	5	-	na	na	Pfcx-	ε3/ε3
C17MS[5] (2178)	m	71	na	17			-	- -	
ε2/ε3									
C18MS (2267)	f	74	sepsis	7	-	-	-		ε3/ε3
C19MS (3026)	m	69	cancer	3	-	-	-		ε3/ε3

[1]Independent PCR assays targeting the 16S rRNA and *omp1* genes of *C. pneumoniae* done on each patient sample. All samples were negative in both assays except for the Pcx sample for patient C1, which was weakly PCR-positive; we confirmed this positivity using the nested amplification systems for *hsp72* and the KDO transferase gene given in Methods. A fully consistent set of samples was not available for the PCR and other analyses described for most patients studied here.

[2]Proximal cause of death, given as listed on autopsy report.

[3]CB, cerebellum; HP, hippocampus; TCx, temporal cortex; Pcx, parietal cortex; Pfcx, prefrontal cortex; na, not available.

[4]*APOE* genotype was determined by the method of Hixson and Vernier (1990).

[5]Patients indicated as MS had multiple sclerosis.

Table 2. Summary of AD patient characteristics and chlamydia-directed PCR results

					PCR[1]				
Patient	Sex	Age at Death	Cause of Death[2]	Time PM (hr)	CB	HP	Tcx[3]	Other	*APOE*[4]
AD1 (3230)	f	82	cardiac arrest	11	-	+	na		ε3/ε3
AD2 (3488)	f	85	cardiac arrest	7	-	+	na		ε3/ε4
AD3 (3449)	f	81	cancer	10	-	-	na		ε3/ε3
AD4 (3432)	m	87	cardiac arrest	6	-	+	na		ε3/ε4

AD5 (3489)	f	77	cancer	15	-	-	na		ε3/ε3
AD6 (9681)	m	68	sepsis	7	-	+	+	Pcx+	ε3/ε3
AD7 (3440)	f	82	cardiac arrest	12	-	+	na		ε3/ε4
AD8 (9658)	f	61	lung carcinoma	23	-	na	+	Fcx+	ε4/ε4
AD9 (8617)	f	78	pneumonia	22	+	na	+		ε2/ε4
AD10 (8714)	f	86	heart failure	11	+	na	+		ε3/ε4
AD11 (9513)	f	70	gangrenous bowel	9	-	na	+	Pcx+	ε3/ε4
AD12 (9150)	f	79	sepsis	16	-	na	+		ε3/ε3
AD13 (9259)	f	70	aspiration pneumonia	24	-	na	+		ε4/ε4
AD14 (9641)	f	87	pneumonia	8	-	+	+		ε3/ε3
AD15 (9352)	f	90	atherosclerosis	9	-	na	+		ε3/ε4
AD16 (144)	m	67	systemic infection	8	+	na	na	Pfcx+	ε3/ε4
AD17 (319)	f	78	respiratory failure	6	-	na	na	Pfcx+	ε3/ε3
AD18 (164)	f	74	pneumonia	3	-	na	na	Pfcx+	ε3/ε4
AD19 (162)	f	78	renal failure	4	+	na	na	Pfcx+	ε3/ε3

[1]Independent PCR assays targeting the 16S rRNA and *omp1* genes of *C. pneumoniae* done on each patient sample; all samples indicated as positive were unequivocally positive in both assays (see Methods).

[2]Proximal cause of death, given as listed on autopsy report.

[3]CB, cerebellum; HP, hippocampus; Tcx, temporal cortex; Pcx, parietal cortex; Fcx, frontal cortex; Pfcx, prefrontal cortex

[4]*APOE* genotype was determined by the method of Hixson and Vernier (1990).

Legend to FIGURE 1.

Ultrastructural analyses of brain tissues from AD patients to identify EB and RB of *C. pneumoniae*. Brain tissues from AD patients PCR-positive for the organism were prepared for EM and IEM analysis; the anti-chlamydial mAb used in the latter analysis was a monoclonal targeting the *C. pneumoniae* MOMP. **Panel A:** typical inclusion body with presumptive EB (arrows) and RB

(arrowheads) as seen in EM analysis of temporal cortex of patient AD8; note that the sizes of the chlamydia-like bodies range from 0.2 μm to 1.0 μm, characteristic for this organism. **Panel B:** similar structure and putative organisms from the temporal cortex of patient AD8 demonstrating inclusions (little arrowheads) in which a typical pear-shaped EB is found (arrow). **Panel C:** IEM analysis of temporal cortex of patient AD8, showing a typical immunolabelled pear-shaped EB characteristic of *C. pneumoniae*. **Panel D:** IEM analysis of the hippocampus of patient AD7, showing RB labelled with the anti-MOMP mAb. **Panel E: Disrupted inclusion demonstrating pleomorphic forms (EB, arrowheads; RB, arrows) of *C. pneumoniae* in the hippocampus of patient AD7; the variability is presumably due to patient-to-patient differences, duration of infection, *etc.* Bars= (A) 0.5μm; (B)1.0μm; (C)0.1μm; (D)0.25μm; (E)1.0μm.**

Legend to FIGURE 2.

Typical immunohistochemical analysis of brain tissues from AD and non-AD control patients using primary monoclonal Ab targeting the *C. pneumoniae* OMP and the chlamydial LPS. **Panel A:** immunolabelling of perivascular cells (small arrows) using the monoclonal anti-LPS antibody; tissue is from temporal cortex of patient AD4. **Panel B:** immunolabelling of apparent glial cells (large arrow heads) in the dentate gyrus of patient AD4, using the anti-LPS mAb. **Panel C:** immunolabelling of tissue from temporal cortex from a non-AD control patient (C17MS, Table 1) using the monoclonal anti-LPS mAb. **Panel D:** immunolabelling of perivascular cells (small arrows) using the monoclonal anti-OMP antibody; tissue is from temporal cortex of patient AD2. **Panel E:** immunolabelling of apparent glial cells (large arrow heads) in temporal cortex of patient AD7, using the anti-OMP mAb. **Panel F:** immunolabelling of tissue from dentate gyrus from a non-AD control patient (C18MS, Table 1) using the monoclonal anti-OMP mAb. Panels A-C,E-F; Bars = 50 μm, and D; Bar=25um.

Legend to FIGURE 3.

Double immunolabelling of AD brain tissues to identify specific host cell types. **Panels A, B:** tissue section from temporal cortex of patient AD4 double immunolabelled for the chlamydial LPS (FITC) (**A**, small arrows) and GFAP (immunoperoxidase) (**B**, large arrows). **Panels C, D:** tissue section from temporal cortex of patient AD4 double immunolabelled for iNOS (FITC) (**C**, arrowheads) and the chlamydial OMP (immunoperoxidase) (**D**, arrows). Bars =50μm.

Legend to FIGURE 4.

Areas of neuropathology in the AD brain show immunoreactivity for *C. pneumoniae* from patient AD7. **Panel A:** Neuritic pathology characteristic of AD was demonstrated with anti-PHF1 monoclonal mAb (small arrows). **Panel B:** Consecutive tissue section from the same sample demonstrating

immunolabelled glial cells infected with *C. pneumoniae* using the monoclonal anti-OMP mAb (large arrows). Bars = 25μm.

Legend to FIGURE 5.

RT-PCR analysis of total RNA from two AD patients, using primers targeting mRNAs from the *C. pneumoniae* KDO transferase and *hsp72* genes. Lanes are: 1, 100 bp size standards; 2, analysis of RNA from cerebellum of patient AD2 for the KDO transferase mRNA; 3, analysis of RNA from cerebellum of patient AD14 for KDO transferase mRNA; 4, analysis of RNA from hippocampus of patient AD2 for the KDO transferase mRNA; 5, analysis of RNA from hippocampus of patient AD2 for *hsp72* rRNA; 6, analysis of RNA from hippocampus of patient AD14 for KDO transferase mRNA; 7, analysis of RNA from hippocampus of patient AD14 for *hsp72* mRNA; 8, negative control amplification (water substituted for nucleic acids, KDO transferase primers); 9, positive control for KDO transferase amplification; 10, positive control for *hsp72* amplification. In Lanes 9-10, genomic

DNA from *C. pneumoniae* strain TW183 (ATCC) was used as template. For patient AD 14, RT-PCR analysis of RNA from cerebellum was negative for the *C. pneumoniae hsp72* gene; RT-PCR analyses for both the KDO transferase and *hsp72* messengers using RNA from temporal cortex of this patient were positive (not shown).